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## **EUROPEAN UNION REFERENCE LABORATORY FOR MARINE BIOTOXINS**

# **Standard Operating Procedure for determination of domoic acid (ASP toxins) in molluscs by UPLC-MS**

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## 1. INTRODUCTION

Domoic acid is a toxin that belongs to the kainoids, a group of amino acids classed as neuroexcitants or excitotoxins that interfere with neurotransmission mechanisms in the brain (Quilliam, 2004). Domoic acid is the causative agent of the Amnesic Shellfish Poisoning (ASP) which can be produced by ingestion of shellfish and other marine organisms containing this toxin.

**Regulation (EC) N° 853/2004** of the European Parliament and of the Council lays down specific hygiene rules for food of animal origin. With regard to the “Health standards for live bivalve molluscs”, it indicates that live bivalve molluscs placed on the market for human consumption must not contain marine biotoxins in total quantities (measured in the whole body or any part edible separately) that exceed 20 milligrams of domoic acid per kilogram for amnesic shellfish poison (ASP).

**Commission Regulation (EC) N° 2074/2005** amended by **Commission Regulations (EC) N° 1664/2006** and **(EC) N° 1244/2007** indicates the recognised testing methods for marine biotoxins for the purpose of Regulations (EC) N° 853/2004 and N° 854/2004. With regard to the ASP detection method it is specified that: “The total content of amnesic shellfish poison (ASP) of edible parts of molluscs (the entire body or any part edible separately) must be detected using the high-performance liquid chromatography (HPLC) method or any other recognised method. However, for screening purposes, the 2006.02 ASP ELISA method as published in the AOAC Journal of June 2006 may also be used to detect the total content of ASP of edible parts of molluscs. If the results are challenged, the reference method shall be the HPLC method”.

Most laboratories use an HPLC-UV protocol for the analysis of domoic acid based on that of Quilliam and col. (1995) with or without the strong anion-exchange column clean-up step. However other chemical methods can be employed for the determination of ASP toxins such as thin layer chromatography, capillary electrophoresis and liquid chromatography coupled to mass spectrometry (Quilliam, 2004).

This Standard Operarion Procedure (SOP) describes a method based on ultraperformance liquid chromatography coupled to mass spectrometry (UPLC-MS) that can be used for the Official Control of domoic acid or as a confirmatory tool to other methods of analysis.

## 2. SCOPE

The method described in this SOP is applicable to the determination of domoic acid in bivalve molluscs. The limit of detection (based on signal/noise= 3) is about 1.5 ng/mL (with the equipment and conditions employed at the CRLMB). The theoretical quantitation limit by this method is (based on signal/noise = 10) is about 5 ng/mL.

DA content is determined by preparing 50% aqueous methanol extracts of the homogenized tissue, filtering and analyzing the extract by ultraperformance liquid chromatography with mass spectrometric detection. Chromatographic separation is performed by gradient elution.

## 3. REAGENTS

- Ultra-pure water.
- Acetonitrile, hypergrade for LC-MS.
- Methanol, hypergrade for LC-MS.
- Formic acid (98-100%).
- Domoic acid certified calibration solution: CRM-DA-f, available from the Institute for Marine Biosciences, National Research Council of Canada, Halifax, Nova Scotia-Canada. The solution has a certified concentration  $101.8 \pm 2.1 \mu\text{g}$  domoic acid (DA) + epidomoic acid (EA)/mL (at 20°C);  $102.7 \pm 2.1 \mu\text{g}$  DA+EA/g. Each ampoule contains 0.5 mL of a solution of domoic acid dissolved in acetonitrile/water, (1:19 v/v). Sealed ampoules should be stored in the dark in a refrigerator (at approximately +4°C). (The solution should not be frozen). Prior

to opening, each ampoule should be allowed to warm to room temperature. Once the ampoule has been opened, accurate aliquots should be removed using calibrated volumetric equipment and transferred to other amber containers for dilution and/or analysis as soon as possible. Closed vials should be stored in the dark in a refrigerator (at approximately +4°C) for no more than 3 months. The concentration of DA in CRM-DA-e is suitable for preparing a dilution series for calibration of LC-MS instrumentation, as well as for spiking shellfish control samples for recovery studies.

- Domoic acid working calibration solutions: Prepare a serie of calibration working solutions with increasing concentration of DA+EA, within the mass range of e.g. 5 to 500 ng/mL, by accurately diluting the certified calibration solution with acetonitrile:water, 1:19 v/v. Keep solutions in the dark and refrigerated (at approximately 4°C) when not in use. Do not store them for more than 3 months. Do not freeze the solutions. Allow the solutions to reach room temperature before use.

#### **4. EQUIPMENT**

Use usual laboratory material and equipment and, in particular, the following:

- Analytical balance
- Blender
- Ultra-Turrax<sup>®</sup> homogenizer (or equivalent)
- Vortex mixer
- Centrifuge capable to reach 3000 x g (refrigerated at aprox. 4°C, if possible)
- Centrifuge tubes with screw tops nominal volume: 30-50 mL
- Adjustable & calibrated automatic pipettes, covering the range from 10 µL to 1000 µL
- Membrane filters: acetonitrile-compatible, pore size 0,2 µm

- Syringe driven membrane filters: methanol-compatible, pore size 0,22 µm
- Plastic syringes 1-2 mL
- Vials suitable for the UPLC equipment
- Glass amber vials, 2 mL or less, with crimp caps (to store domoic acid working calibration solutions)

### UPLC-MS EQUIPMENT

Acquity UPLC<sup>®</sup> system equipped with a sample manager, a binary solvent manager and a high temperature column heater, from Waters. The MS analysis is performed in a TQ detector (Waters).

Column: Acquity UPLC<sup>®</sup> BEH C18 1.7 µm, 2.1 x 50 mm

## **5. PROCEDURE**

### **5.1 Sample preparation**

**Bivalves with shell:** Thoroughly clean outside of the shellfish with fresh water. Open by cutting adductor muscle. Rinse inside with fresh water to remove sand and foreign material. Remove meat from shell by separating adductor muscles and tissue connecting at hinge. Do not use heat or anaesthetics to open the shell. After removal from shellfish, drain tissues 5 min in a sieve to remove salt water. For representative sampling, at least 100-150 g of pooled tissue should be homogenized in a grinder or blender. Subsamples from this homogenate can be taken immediately after blending, while still well mixed, or after mixing again. In the case of scallops, at least 10 specimens or individual edible parts (muscle, muscle + gonad) should be taken should be taken (Commission Decision 2002/226/CE).

**Bivalves without shell (whole body or any edible part separately):** If needed, clean outside with fresh water and allow it to drain. For representative sampling, at least 100-150 g of pooled tissue should be homogenized in a grinder or blender. Subsamples from this homogenate can be taken

immediately after blending, while still well-mixed, or after mixing again. In the case of scallops, at least 10 specimens or individual edible parts (muscle, muscle + gonad) should be taken (Commission Decision 2002/226/CE) (8).

## **5.2 Extraction procedure**

Accurately weigh  $4 \pm 0.1$  g of tissue homogenate into a graduate centrifuge tube or a stainless steel micro-blender cup. Add 16 mL of extraction solvent (methanol:water 50:50) and homogenize the sample extensively (3 min. at 10000 rpm). Do not try to recover all the tissue remaining in the homogenizer probe or blender cup, but wash them thoroughly afterwards to prevent contamination of the next sample.

If a blender has been used for homogenization, pour the resulting slurry into a centrifuge tube.

Centrifuge at  $3000 \times g$  or higher for 10 min. Filter a portion of the supernatant through a methanol-compatible  $0,22 \mu\text{m}$  filter. If you suspect from a positive sample, or if you work with a spiked sample it is required to dilute the sample extract before injecting it (i.e. dilution 1/10). Sample extracts should be analyzed as soon as possible and should be maintained refrigerated while not injected. If analysis is not performed immediately, the extract may be stored in a tightly sealed screw-capped storage container in a freezer at c.a.  $-18^{\circ}\text{C}$ .

Extraction blank: perform the extraction procedure (see above) except substitute 4 g of water in place of sample tissue (chromatograms should be free of peaks eluting near domoic acid or causing excessive baseline slope).

## **5.3 UPLC/MS determination**

The following conditions have been proved as suitable for DA analysis at the CRLMB.

UPLC conditions for the analysis of domoic acid:

Column	Acquity UPLC <sup>®</sup> BEH C18 1.7 $\mu$ m, 2.1 x 50 mm
Column oven T <sup>a</sup>	35°C
Mobile Phase	<b>A:</b> 100% H <sub>2</sub> O with 30mM formic acid
	<b>B:</b> 95% ACN: 5% H <sub>2</sub> O 30mM formic acid
Flow	400 $\mu$ L/min
Run time	3.0 min
Injection volume	5 $\mu$ l
Sample manager T <sup>a</sup>	7°C

Elution was achieved with gradient conditions:

Time (min)	% A	% B	Curve
Initial	95	5	--
0.30	95	5	6
2.50	60	40	6
2.51	95	5	6
3.00	95	5	--

MS Instrument Parameters:

Polarity: Positive

Capillary (kV) 3.00; Cone (V) 35.00; Extractor (V) 3.00; RF (V) 0.10

Source Temperature (°C) 120; Desolvation Temperature (°C) 300;

MSMS Mode Collision Energy 18.00

Detection mode: Multiple Reaction Monitoring (MRM): Precursor ion: 312.2 *m/z*, Product ions: 266.2; 248.2; 193.2. Quantification trace: 312.2>266.2; Secondary ion trace 312.2>248.2 (used to calculate ion ratio). Additional confirmatory trace: 312.2>193.2.

Masslynx software was used for MS tune, instrument control, data acquisition and data analysis.

## **6. CALIBRATION AND QUANTIFICATION**

### **6.1. Calibration graph and sample injection**

Prepare a calibration graph each day of analysis. Inject domoic acid working calibration solutions over the range of e.g. 5 to 500 ng/mL. Standards and samples are injected in triplicate.

Plot the peak area against the concentration of the injected DA+EA calibration solutions. Ensure that the coefficient of correlation of calibration curve shows a linear regression ( $r > 0.99$ ).

Integrate DA+EA areas in each sample injection and calculate average peak areas (DA+EA) for each sample. Determine each sample concentration (DA+EA) with the calibration graph (external standard method).

### **6.2 Identification**

Identify the presence of domoic acid by comparing the peak retention time of the suspected domoic acid in the sample with that of the standards.

The use of two ions for the MS detection is recommended to obtain a high degree of specificity. In this method the secondary ion trace 312.2>248.15 was used to calculate the ion ratio. An additional confirmatory trace employed was: 312.2>193.15.

### **6.3. Quantification**

Quantification trace: 312.2>266.15.

After determining each sample concentration (DA+EA), (external standard method) with the calibration graph, calculate the level of domoic acid + epidomoic acid in the sample using the following formula:

$$\text{mg DA+EA/kg sample} = \frac{\mu\text{g DA+EA / mL injected extract} \times V_t (\text{mL}) \times D}{W (\text{g})}$$

*V<sub>t</sub>* = Total volume of homogenate and extracting solvent in mL

*W* = Sample tissue weigh (usually 4 g)

*D* = Dilution factor (if extract has been diluted)

## 7. PRELIMINARY RESULTS OBTAINED WITH SOME SAMPLES

Sample type	Results obtained with analysis by UPLC-MS (µg DA+EA/g sample)	Recovery (%)	Results obtained with analysis by HPLC-UV (µg DA+EA/g sample)
Blank mussel spiked with mussel reference tissue (theoretical concentration: 19.93 µg DA+EA/g)	21.83	109.55	19.59
Blank mussel spiked with mussel reference standard (theoretical concentration: 3.50 µg DA+EA/g)	3.83	109.43	3.63
Scallop, naturally contaminated, from previous proficiency test (assigned value: 34.84 µg DA+EA/g)	38.93 (z-score from lab. with similar result: 0.995)	---	38.5 (z-score: 0.876)
Scallop, naturally contaminated, from previous proficiency test (assigned value: 49.41 µg DA+EA/g)	54.13 (z-score from lab. with similar result: 0.949)	---	50.25 (z-score: 0.170)
Blank mussel	< L.Q.	---	< 1.8 µg DA+EA/g
Blank clam	< L.Q.	---	< 1.8 µg DA+EA/g
Blank scallop	< L.Q.	---	< 1.8 µg DA+EA/g
Blank razor-clam	< L.Q.	---	< 1.8 µg DA+EA/g

L.Q.: theoretical quantitation limit

## 8. REFERENCES

- Commission Decision 2002/226/EC of 15 March 2002 establishing special health checks for the harvesting and processing of certain bivalve molluscs with a level of amnesic shellfish poison (ASP) exceeding the limit laid down by Council Directive 91/492/EEC. DOCE L 75 of 16.3.2002, pp. 65-66.
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- Regulation (EC) N° 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. DOCE L 226 of 25.06.2004, p. 22-82.